

*On the Identification of Chitin by its Physical Constants.*

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The determination of the distribution of chitin in the animal kingdom is hampered by the absence of any test or positive means of identifying it. Gamgee, in his 'Text-book of Physiological Chemistry,' gives a list of structures of invertebrate animals in which chitin has been described. But when those cases are eliminated in which the identification has been based solely on the negative character of insolubility in caustic alkalis or weak acid, the revised list, as it appears, for instance, in von Fürth's 'Vergleichende chemische Physiologie der niederen Tiere,' is greatly curtailed. It is true that chitin yields a characteristic decomposition product, the amido-derivative of sugar known as chitosamin, in definite proportions, but the amount of material available is not always sufficient to allow of the preparation of this product. In cases, however, where we have other reasons for suspecting the presence of chitin, the reducing action of the chitosamin resulting from the treatment of the original substance with sulphuric acid is a valuable confirmatory test.

Under these circumstances it seemed that it might be worth while to make a determination of the specific gravity of chitin by the well-known method of a diffusion column. The specific gravity of any substance as thus determined stands in real and intimate relation to its chemical constitution, for the mass dealt with is always small and can therefore be rendered homogeneous by various cleansing processes. It is readily permeated by the suspending fluid and the absence of any other sources of error may be ensured without difficulty.

In addition to the determination of the specific gravity, that of the refractive index should be made, for two substances may possess the same specific gravity, but not the same chemical composition, though when we have other reasons for suspecting their identity this is unlikely.

The refractive index of any substance has been shown by Gladstone and others to be connected with the specific gravity by a relation which, whether expressed as  $\frac{n-1}{d}$  or more correctly as  $\frac{n^2-1}{(n^2+2)d}$  is constant for that substance and is independent of the temperature. Therefore the demonstration

of common values for the specific gravity and the refractive index of two substances affords strong evidence of their identity.\*

Unfortunately, I do not possess a reading microscope, so that I have taken my readings direct from a scale at the back of the experimental tube. But I have appreciated the advantage of the use of glass beads: when these are made and suitable fluids chosen the process need occupy but little time.

The fluids I have used are chloroform, of which the specific gravity is roughly 1.48 at 21° C., and "absolute" alcohol of specific gravity 0.791 at 21° C. The specific gravity of chitin from the integuments of *Astacus* having been found roughly to lie somewhere between 1.39 and 1.41, mixtures of chloroform and alcohol of specific gravity 1.36 and 1.44 were made with the help of a Westphal's balance, and these mixtures formed the two fluids of the column. In using light fluids it is not possible to float glass beads, such as are used for dealing with minerals in heavy fluid columns, and which are described in 'Nature':† it is necessary in the present case to blow the beads dumbbell-shaped, but with both ends hollow. Hence it is possible to obtain beads which float with their long axis horizontal and this is an advantage, for the nearer the axis of the bead approaches to the horizontal position the less is likely to be the error which arises in estimating its centre of gravity.

Chitin from various sources was prepared in the usual way by soaking in 10-per-cent. hydrochloric acid, boiling in 5-per-cent. caustic potash‡ for many hours with frequent changes of the liquid, washing in water, very dilute hydrochloric acid, and extracting in alcohol and ether. In some cases this treatment did not render the chitin quite colourless: the last traces of pigment were removed by Mayer's method of bleaching, in which chlorine is the active agent.

The specific gravity of chitin from integuments of *Astacus* cleaned in this way was found to range between 1.400 and 1.404. The chitin of a caterpillar, *Bombyx cynthia*, was slightly heavier, 1.404 to 1.408, of the cockroach wing 1.402, of the pupa case of the buff-tip moth 1.404: of *Oniscus* and spiders, again, the value agreed very nearly with that of *Astacus*. Two species of *Myriapoda* gave the value 1.397 to 1.399; one of these was the common English *Lithobius*, the other a large millepede which I picked up in the Karroo, South Africa, but have not identified.

\* This method was originally published in 1885 by Professor Sollas in the 'Roy. Soc. Dublin Proc.,' new ser., vol. 4, p. 378, and subsequently in 'Nature,' 1891, vol. 43, p. 404, and 'Quart. Journ. Geol. Soc.,' vol. 58, p. 163, 1902.

† *Loc. cit.*

‡ The use of potash of this strength, which is weaker than that employed by Krukenberg, was recommended to me by Dr. F. Gowland Hopkins, to whom I am indebted for much kindly interest in this investigation.

The chitin which had the lowest specific gravity of any I have measured was that of *Limulus* and of *Scorpio*. This had a value 1.393 when cleaned as above described. If, however, stronger potash is used, chitin from *Astacus* and from *Limulus* approach each other in a diffusion column and give a zone at a level corresponding to a specific gravity 1.398. This figure seems to represent the value of the specific gravity of pure chitin, for, as stated below, it is that of chitin precipitated from its solution in strong acid.

The strength of potash which gives a common value for the specific gravity of chitin from both *Limulus* and *Astacus* is 40 per cent. It must be used for a short time only after the usual long boiling in 5 or 10 per cent, as the prolonged action of strong potash causes chitin to become soluble in weak acid.

Neither boiling in 50-per-cent. hydrochloric acid nor soaking in strong hydrofluoric acid alters the specific gravity of chitin.

Young chitin from a crayfish which had recently undergone ecdysis was heavier than the older skin; it formed a zone at a level corresponding to a specific gravity of 1.410, with outlying pieces down to 1.420. Unfortunately, I have not obtained the specific gravity of the cast skin. It proved to be more readily soluble in weak acid after boiling potash than the younger chitin and, not foreseeing this, I inadvertently dissolved it before I had taken measurements.

The refractive indices for red light of the chitin from various sources which I have measured lie between the limits 1.550 and 1.557. The measurement was made by Becke's method.\* That from *Astacus* and *Limulus* integuments lies between 1.554 and 1.557. The fluids used in these determinations were aniseed oil and mixtures of aniseed and fennel oils calculated to have the refractive indices 1.550 and 1.557.

As stated above, chitin was precipitated from its solution in strong hydrochloric acid, in order to determine which of the obtained values, ranging between the extremes 1.393 and 1.408, represents the specific gravity of pure chitin.

The solution was performed in a refrigerator, carapaces of *Astacus* and *Limulus* being used. At the end of a fortnight no difference in the bulk of the solid was noticeable on inspection. The acid was then poured off and diluted in the refrigerator with 10 times its volume of water cooled till a thin film of ice formed on the surface. A copious milky precipitate was formed. Fresh acid was poured over the remaining pieces of chitin, and slight precipitates obtained at intervals of two days from this freshly

\* "Ueber die Bestimmbarkeit der Gesteinsgemengtheile auf Grund ihres Lichtbrechungsvermögens," 'Sitzungsber. der K. Akad. der Wissensch.,' Vienna, vol. 102, p. 358, 1893.

added acid. The solution in hydrochloric acid, when kept at a temperature of less than  $0^{\circ}$  C., is thus seen to be not unstable. The precipitate, which was flocculent, was washed by decantation until an acid reaction was no longer obtained; it was then boiled in alcohol and ether. Placed in a specific gravity column, it formed a broad zone, extending from 1.380 to 1.390. Some of the precipitate was then dried at a low temperature ( $40^{\circ}$  to  $60^{\circ}$  C.). It formed a thin film on the evaporating dish, which was scraped off with a scalpel, and then formed a white powder. This powder had a specific gravity 1.398, and this, I conclude, is the specific gravity of chitin. If dried too rapidly at a higher temperature than  $60^{\circ}$ , the precipitate flakes off the dish and its specific gravity is no longer uniform.

The substances of unknown chemical composition which I have examined by this method are *Lumbricus* bristles, the organic portion of the molluscan radula, the pupal skin of *Pieris*, and of some other Lepidoptera stated by Griffiths to be "pupin." I had hoped to deal also with the skeletal substance of the branchial bars of *Amphioxus*, which are stated to be insoluble in alkalis, but I could obtain no residue after boiling the pharynx, even in weak caustic potash. An account of the specific gravity of the organic portion of the shell of *Sepia*, in which chitin has been found by Krukenberg, is also given below. *Lumbricus* chaetæ were isolated in the following manner:—The worm was slit up along the mid-dorsal line and the alimentary canal and its contents removed. The remaining body wall was then washed in a current of water to remove any matters which might have escaped in the process of removal of the gut. The contents of the gut are troublesome unless removed at once. The worms thus far cleaned are now treated with 10-per-cent. hydrochloric acid, and then boiled in potash as usual in the preparation of chitin. If after prolonged treatment with potash any matter other than chaetæ remains undissolved, this is most readily got rid of at a later stage of the cleansing process (after extraction in alcohol) by separation in a diffusion column, say of chloroform and alcohol. The bristles will float on the chloroform, the insoluble dirt (presumably sand, undigested wood fibres, and cellulose, set free in removing the alimentary canal) sinks to the bottom. The bristles can then be removed with a pipette and extracted in ether. In this way a considerable quantity of chaetæ can be collected with very little trouble. They form a neat zone in a diffusion column at a level corresponding to a specific gravity 1.392. They are doubly refracting. I have measured one index only, it lay between 1.557 and 1.550. I conclude, therefore, that the chaetæ of *Lumbricus* contain chitin. This is in agreement with the results obtained by Goodrich.\*

\* 'Quart. Journ. Micr. Sci.', 1897, vol. 39, "Notes on Oligochaetes."

From their solubilities and their behaviour with Millon's reagent and with the xanthoproteic test, Goodrich considered that the bristles of Oligochaeta are evidently chitin or some nearly allied substance. But more recently Schepotieff,\* after a lengthy research, concludes: "Diese Reaktionen zeigen, dass die Borsten jedenfalls nicht aus einer einheitlichen Substanz bestehen, sondern aus mindestens zwei. Die eine derselben zeigt die Reaktionen der Eiweisskörper. Die andere kann schwerlich Chitin sein, wie der Mangel der Zuckerbildung bei Behandlung mit Schwefelsäure ergiebt." With regard to the last difficulty, the absence of sugar formation after treatment with sulphuric acid, I am unable to find that it exists, for I obtained copious sugar reduction with Trommer's test. Schepotieff (p. 672) dissolved the bristles in 89-per-cent. sulphuric acid, kept them for 25 hours at a temperature of 40° to 50° C., diluted the solution with 10 times its bulk of water, and kept the diluted solution at 100° C. for some hours. He then neutralised with barium carbonate, and tested the solution with copper sulphate and sodium hydrate. It was suggested to me by my friend Miss Durham that the use of barium carbonate in place of the usual caustic potash for neutralisation may have led to Schepotieff's difficulty, as the precipitate, consequent on neutralisation with barium carbonate, would carry down the sugar with it. At any rate, sugar is certainly present after treatment with sulphuric acid, and there is no reason why one of the two constituents should not be chitin. That more than one substance should be present in the bristles is not surprising. I have not met with any chitinous skeleton which did not give the proteid reaction to which Schepotieff alludes before it was cleaned.

Pupa cases of *Pieris brassicae* and *P. napi*, cleaned in the same way as chitin, had a specific gravity of 1.400 and refractive index between 1.554 and 1.557. Griffith† has examined these integuments chemically, and has stated that they consist of a new animal substance which he calls "pupin." He dissolved the skins (after they had been cleaned with potash, water, alcohol, and ether) in hydrochloric acid, and precipitated from this solution a substance to which he assigned the formula  $C_{14}H_{20}N_2O_5$ , and found that it split when boiled with strong mineral acids into leucin and carbon dioxide. Von Fürth remarks that the proportions of C, H, and O point to an albuminoid, but the N-content is strikingly small. He suggests that a closer investigation would be worth while, as he supposes that the products of splitting, besides leucin and carbon dioxide, must have been overlooked.

\* 'Zeitschr. Wiss. Zool.,' vol. 74, 1903, p. 674.

† 'Comptes Rendus,' II, vol. 105, pp. 320—321, and 'Bull. Acad. Roy. Belg.,' (3), vol. 24, 1892, p. 592.

I can only state that the substance has, like the integument of other Lepidopteran pupæ, the physical properties of chitin, and that I have obtained evidence of the presence of sugar in it after treatment with 90-per-cent. sulphuric acid at a temperature of 40° C. for 24 hours. The sugar was detected by Trommer's test, as in the other cases mentioned.\* The molluscan radula I have studied from this point of view by this method chiefly in the case of the periwinkle, *Littorina littorea*, the limpet, *Patella vulgata*, and the whelk, *Buccinum undatum*. The radula of *Patella*, as I have shown elsewhere,† contains silica and iron in considerable quantities. To cleanse it we must, therefore, treat it for some time (24 hours) with strong hydrofluoric acid. A control experiment showed that the specific gravity of chitin from the integument of *Astacus* was unaltered by this treatment. After washing out the hydrofluoric acid the usual method of preparing chitin was used. The radulae still retain a faint yellow colour after this treatment; they were placed in chlorine water, when they became colourless and somewhat transparent. They were cut into short lengths, and these were found to form a neat zone in the diffusion column at a level corresponding to a specific gravity 1·405 to 1·407. *Littorina* radulæ gave a closely similar result. In the case of *Littorina* I used the confirmatory test, and obtained reduction of copper salts by Trommer's method.

*Buccinum* radulæ differ from the others which were measured in that they form a broad irregular zone in a diffusion column. The extreme tips of the radulæ, taken from the radular sac, however, give a uniform specific gravity of 1·404. The remainder of the radulæ form a band extending from 1·400 to 1·411, or even lower. It is noticeable that the pieces in this band for the most part hang with their greater length vertical, and the oldest end of each length is lowermost. It, therefore, seems possible that some second resistent substance, in addition to chitin, is present in this case in increasing quantities as we pass forwards from the radular sac. If this is the correct interpretation of the facts, it is interesting in connection with the occurrence of free sulphuric acid in the salivary glands of *Buccinum*, and it is not surprising that an organ used to bore holes in shells with the aid of sulphuric acid should need to be specially resistent. For some time I fancied that the irregularities in the specific gravity of this radula might be due to small particles of sand caught between the bases of the teeth and the basal membrane. But the specific gravity of the teeth, when isolated

\* Since this was written, von Fürth and Russo have shown by chemical methods that the integuments of Lepidopteran pupæ consist of ordinary chitin, 'Beitr. z. chem. Physiol. u. Pathol.', vol. 8, 1906.

† 'Quart. Journ. Micr. Sci.', 1907, vol. 51, pp. 115—136.

from the basal membrane, shows that this cannot be so. The same radulae of which I had measured the specific gravity were treated for a moment with strong hydrochloric acid, with the result that the basal membrane was instantly destroyed and the teeth freed; after washing and reboiling in alcohol they were found to have specific gravities of even wider range than that of the intact radula, the heaviest of them being 1.430. By soaking the radula of *Buccinum* for several weeks in very strong potash (above 40 per cent.) the teeth can be isolated, the basal membrane being destroyed. This fact, discovered by chance, aroused some doubt as to whether the basal membrane were really chitin, and a repetition of the experiment was made in which the skeletons of two segments of *Astacus* were subjected to the action of the same strength of potash. The two segments became isolated from one another, the abdominal feet (pleopoda) were also isolated from the segments, the soft connecting membranes being destroyed. And yet these membranes have the same specific gravity and the same refractive index as chitin, and no one has ever suggested that they are anything else. These membranes differ in staining reactions from the chitinous pieces which they connect in just the same way as the basal membrane of *Buccinum* differs from the teeth.

A third method of isolating these teeth is to soak the radula in hydrofluoric acid for 24 hours previous to the usual cleansing process. The teeth will then be liberated when the radula is boiled in 5-per-cent. potash solution. Teeth thus prepared gave the least range that I have found in this material; they formed in all cases a dense zone at the level 1.393; in one instance they were confined to this zone, but in two other columns a certain number of teeth were scattered down to 1.411, and a very few, perhaps five or six, hung at various depths below this. *Astacus* integuments subjected to precisely the same treatment had a specific gravity of 1.406; and, as happened when strong potash was used, the connecting membranes were destroyed. Some of the heavier *Buccinum* teeth were removed with a pipette from the column; in one or two of them a single large granule, apparently a foreign body, was visible: it appeared to have undergone partial solution. Since, in the case of *Murex*, I have seen obvious sand grains firmly embedded in the teeth of the radula, it would seem probable that the exceptionally heavy teeth are to be accounted for by accidents of this kind. The minor variations in specific gravity may perhaps be due to some second organic substance accumulated in the thick body of the tooth remote from the margin, and thus not affecting the measurement of the refractive index, which lies constantly between 1.550 and 1.554. The fact that the heavier teeth hang in the column with their fangs upwards is in accordance with this view.

The refractive indices of the organic portion of the Molluscan radula,

which I have determined in *Littorina*, *Patella*, and *Buccinum*, lie between 1.550 and 1.554.

The shell of *Sepia* has been shown by chemists to contain chitin, and the percentage yield of chitosamin has been measured by them. After decalcification with 10-per-cent. hydrochloric acid and boiling in 5-per-cent. potash it readily breaks up, the thick outer wall of the shell with the rostrum and lateral expansions becoming freed from the mass of delicate septa. This outer layer quickly becomes transparent and colourless in parts, the rostrum and thickened region in its neighbourhood being yellowish. The septa remain opaque, they adhere to one another by a maze of low sinuous ridges, the capillary spaces between which readily become filled with air, unless special care is taken to avoid this. The specific gravity of the outer layer is not a constant quantity, but ranges from 1.385 to 1.393. Alternate boiling in various strengths of potash higher than 5 per cent. and in 50-per-cent. hydrochloric acid do not reduce this range. The septa, on the other hand, form a zone in the diffusion column at the level of chitin. The refractive index of the inner laminæ lies between 1.550 and 1.554; that of the outer is variable, lying sometimes between 1.550 and 1.544, sometimes between 1.550 and 1.554. The variable specific gravity of the outer layer of the *Sepia* shell points to the presence of more than one substance. As the change in specific gravity goes hand in hand with a corresponding change in refractive index, the suggestion naturally arises that there may be a series of chitins, of which a lighter and a heavier member are present in the *Sepia* shell.

Rosenheim\* has given some reason to suppose that the fossil carapace of *Pterygotus osiliensis* contains chitin. It would be interesting to apply the method described in this paper in confirmation of his results, and to extend it to the study of other organic substances.

#### Summary.

The determination of the physical constants of chitin forms a useful method of identifying it. The specific gravity of chitin from various sources approximates to the value 1.398, a number which represents the specific gravity of chitin precipitated from its solution in strong acid. The refractive index lies between the limits 1.550 and 1.557.

The bristles of *Lumbricus*, the pupal skin of *Pieris* and other Lepidoptera, the radula of Mollusca and the shell of *Sepia*, when freed from mineral matter and easily soluble organic substances, have specific gravities and refractive indices which lie between the same limits as those of chitin from various sources.

\* 'Roy. Soc. Proc.,' B, vol. 76, 1905, pp. 398—400.